TOXICOLOGICAL SCIENCES **78,** 187–195 (2004)
DOI: 10.1093/toxsci/kfh066
Advance Access publication January 21, 2004

Epoxide Hydrolases in the Rat Epididymis: Possible Roles in Xenobiotic and Endogenous Fatty Acid Metabolism

Shelley Brown DuTeaux,* John W. Newman,† Christophe Morisseau,† Elise A. Fairbairn,*[1] Karen Jelks,*[2] Bruce D. Hammock,† and Marion G. Miller*,[3]

Department of Environmental Toxicology and †Department of Entomology and the UC Davis Cancer Center, University of California-Davis, Davis, California 95616

Received November 14, 2003; accepted December 10, 2003

Epoxide hydrolases play an important role in detoxifying epoxides that arise from the metabolism of xenobiotic and endogenous compounds. Both the soluble and microsomal forms of epoxide hydrolase (sEH and mEH, respectively) have been detected in the rat testis. Because of the important role the epididymis plays in sperm maturation and protection, the present study evaluated the presence and activity of these two epoxide hydrolases in the rat epididymis. Using Western blotting, protein bands consistent in size with both mEH and sEH were detected in the caput, corpus, and cauda of the epididymis. The mEH immunoreactive bands in the epididymis (~50 kDa) were consistent with mEH detected in the liver and kidney. The sEH immunoreactive bands in the epididymis (~65 kDa) were consistent with a recombinant sEH standard and sEH detected in the liver, kidney, and testis. The presence of mEH and sEH in the epididymis was supported by observations from substrate-based enzyme assays. Results indicated that epididymal mEH can hydrolyze [^{3}H]-cis-stilbene oxide to the corresponding diol at levels ~9% of the kidney. Epididymal sEH hydrolyzed the substrate [^{3}H]-trans-diphenylpropene oxide to the corresponding diol and this activity was inhibited by cyclohexyl-dodecyl urea. Arachidonic acid epoxygenase activity was detected in epididymal S9 fractions, suggesting that fatty acid metabolism by epididymal cytochrome P450s can form epoxides that subsequently become substrates for epididymal sEH. Results from the present study indicate that the epididymis contains at least two active forms of epoxide hydrolase. The role of these enzymes in the detoxification of xenobiotic epoxides is well known, although it is unclear what cellular role they may play in the formation of biologically active metabolites in the epididymis.

Key Words: **arachidonic acid; epididymis; epoxide hydrolase; metabolism.**

The epididymis is the organ within which sperm undergo the final stages of maturation, gaining motility and the ability to fertilize oocytes *in vivo* (Amann *et al*., 1993). Along with the processes of sperm maturation, the epididymis may play a role in protecting sperm during epididymal transit (Hinton *et al*., 1995). Previous reports have indicated that the epididymis contains glutathione S-transferases (GSTs), cytochrome P450 2E1 (CYP2E1), and alcohol dehydrogenase (DuTeaux *et al*., 2003; Tietjen *et al*., 1994; Veri *et al*., 1994). The presence of these enzymes supports the notion that the epididymis may play a role in the detoxification of xenobiotic compounds *in situ*. Alternatively, these enzymes can bioactivate certain chemicals, producing reactive and potentially more toxic intermediates from the parent compound (Yost, 2001). The relative amount and activity of bioactivating versus detoxifying enzymes can greatly influence the likelihood of toxicity in a target organ (Yost, 2001).

Epoxides are a class of compounds that can arise from the cytochrome P450–mediated oxidation of alkenes, aromatic hydrocarbons, heterocycles, and vinyl halides that exist in the environment (Guengerich, 2003). Cytochrome P450s can also form lipid epoxides from endogenous arachidonic acids (Chacos *et al*., 1983). The presence of CYP2E1 in the epididymis may provide the biochemical pathway by which this organ can potentially form epoxides from exogenous substrates (DuTeaux *et al*., 2003). Many epoxides, which may be considered mutagenic, carcinogenic, and cytotoxic (Hammock and Ota, 1983), can be transformed and/or detoxified by two major pathways. GSTs mediate the conjugation of epoxides with glutathione (Chasseaud, 1979). Epoxide hydrolases catalyze the addition of H_2O across the epoxide to form the corresponding diol (Lu and Miwa, 1980). The resulting products are typically less reactive, more water soluble, and more easily excreted from the body (Guengerich, 2003). As such, the enzymatic transformation of epoxides is generally considered protective (Gill *et al*., 1983).

The two most abundant epoxide hydrolases are the microsomal (mEH) and soluble (sEH) forms (Hammock and Ota, 1983). The microsomal form has a metabolic preference for

[1] Present address: Davis Bodega Marine Laboratory, University of California-Davis, Davis, California 95616.

[2] Present address: Department of Molecular Biosciences, Davis School of Veterinary Medicine, University of California-Davis, Davis, California 95616.

[3] To whom correspondence should be addressed at Department of Environmental Toxicology, University of California-Davis, Davis, California 95616. Fax: (530) 752-3394. E-mail: mgmiller@ucdavis.edu.

arene oxides and exhibits broad substrate specificity for mutagenic and toxic xenobiotic epoxides (Hammock and Ota, 1983). Metabolically active mEH has been detected in several rat organs including the liver, kidney, and testis (Draper and Hammock, 1999; Hammock and Ota, 1983; Moody *et al*., 1986). In addition to its detoxifying role, mEH is hypothesized to be involved in bile acid transport (von Dippe *et al*., 1996). Like mEH, sEH has been detected in the mammalian liver, kidney, and testis (DiBiasio *et al*., 1991; Moody *et al*., 1986). However, sEH has a higher affinity for aliphatic epoxides and is considered less efficient in catalyzing the hydrolysis of certain epoxides such as 1,2-naphthalene oxide (Wang *et al*., 1982).

In addition to metabolizing exogenous epoxides, sEH can hydrolyze lipid epoxides that arise from the cytochrome P450–dependent metabolism of arachidonic acids (Fig. 1; Chacos *et al*., 1983; Fang *et al*., 1997; Yu *et al*., 2000). Lipoxygenases produce various mid-chain hydroperoxides, which are reduced by glutathione peroxidase yielding the corresponding hydroxy eicosatetraenoic acids (HETE). These mid-chain HETEs have numerous roles in inflammatory and proliferative responses (Roman, 2002). Hydroxylation of the omega-carbon (C20) of the arachidonic acid chain by cytochrome P450 4A (CYP4A) yields 20-HETE. This compound inhibits calcium-dependent potassium (K^+_{Ca}) channels, preventing cellular hyperpolarization (Zou *et al*., 1996). The action of 20-HETE is opposed by the cytochrome P450 generation of epoxides of arachidonic acid, i.e., epoxy eicosatrienoic acids (EETs). These compounds increase the open state probability of K^+_{Ca} channels (Campbell *et al*., 1996). The 8,9-, 11,12-, and 14,15-EETs can be transformed by sEH to their corresponding dihydroxy eicosatrienoic acids (DHETs; Newman *et al*., 2002). Certain DHETs have an equipotent or greater ability to activate K^+_{Ca} channels than their EET precursors (Lu *et al*., 2001). Therefore, the enzyme activity of sEH may enhance the biological effect of certain arachidonic acid metabolites.

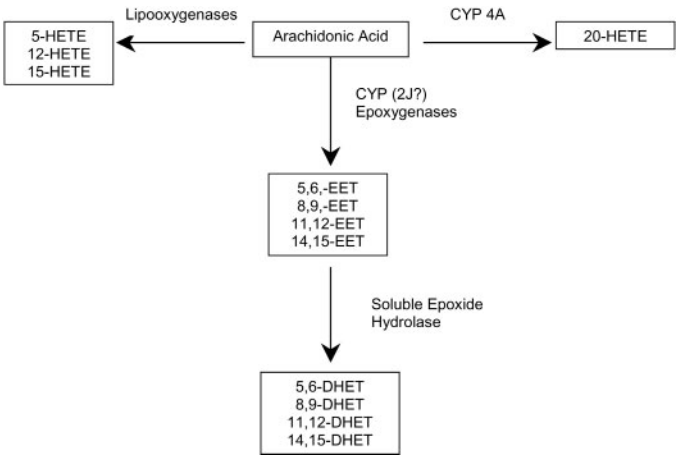

FIG. 1. The proposed pathways of arachidonic acid metabolism. CYP 4A, cytochrome P450 4A; CYP 2J, cytochrome P450 2J; DHET, dihydroxy eicosatrienoic acid; EET, epoxy eicosatrienoic acid; HETE, hydroxy eicosatetraenoic acid.

The objective of the present study was to investigate the presence and activity of the microsomal and soluble epoxide hydrolases in epididymal tissue using immunochemical techniques and enzymatic assays. The possibility that clofibrate could induce sEH activity in the epididymis, as it does in the liver (Hammock and Ota, 1983), was also studied. Finally, to test the hypothesis that P450-dependent fatty acid metabolism could provide endogenous substrates for sEH hydrolysis, we examined the formation of arachidonic acid epoxides in the epididymis.

MATERIALS AND METHODS

Animals. Sexually mature male Sprague Dawley rats (350–400 g) were purchased from Charles River Laboratories (Hollister, CA) and housed with a 12 h light/dark cycle in a temperature- (22 ± 2°C) and humidity- (40–70%) controlled facility. Rats were maintained on Formulab Purina 5008 rat chow (Purina, St. Louis, MO) *ad libitum* and deionized water treated by reverse osmosis. The use of pesticides and chemicals was prohibited in the animal rooms for the duration of the experiment. The University of California-Davis, Animal Use and Care Administrative Advisory Committee approved all animal use.

Chemicals and antibodies. All chemicals and reagents were purchased from either Aldrich (Milwaukee, WI) or Sigma Chemical Co. (St. Louis, MO) and used without further purification unless otherwise specified. A polyclonal antibody against recombinant murine sEH was prepared in the Hammock laboratory, as described previously (Takashi *et al*., 2002). Polyclonal mEH antibody was purchased from Oxford Biomedical (Oxford, MI). Alkaline phosphatase–conjugated antidonkey and antigoat IgG antibodies were purchased from Promega (Madison, WI). Arachidonic acid was purchased from NuChek Prep (Elysian, MN). Oxidized lipids for quantitative analysis were synthesized, purchased, or provided by collaborators. Hydroxyeicosatrienoic acids (i.e., HETEs) were purchased from Caymen Chemical (Ann Arbor, MI). The internal standard, 20-hydroxyecosanoic acid, was purchased from Larodan Fine Chemicals (Malmo, Sweden). The analytical surrogates [10(11)-epoxyheptadecanoic acid and 10,11-dihydroxynonadecanoic acid], linoleate-derived metabolites, and epoxy eicosanoids were synthesized and purified as previously described (Falck *et al*., 1990; Greene *et al*., 2000; Gunstone and Schuler, 1975; Newman *et al*., 2002). Purified arachidonic acid–derived diol (i.e., DHET) regioisomers synthesized in the laboratory of Dr. J. R. "Camille" Falck were a kind gift from Dr. Darryl C. Zeldin at the National Institute of Environmental Health Sciences (Research Triangle Park, NC). The sEH inhibitor cyclohexyl-dodecyl urea was synthesized in the laboratory as previously described (Morisseau *et al*., 2002).

Subcellular fractionation. Rats were sacrificed by CO_2 inhalation and the epididymides, testes, liver, and kidney were dissected. Subcellular fractions were prepared by mincing tissues and homogenizing by hand in a 1:3 ratio of tissue to buffer (0.1 M sodium phosphate buffer [pH 7.4], 0.1 M phenylmethylsulfonyl fluoride, 50 mM EDTA, 1.5 g/l DTT). Kidneys and reproductive tissues were disrupted for 15 sec using a motor-driven pestle. All homogenates were centrifuged at 9000 × g for 20 min at 4°C. Portions of the 9000 × g fractions (S9) were retained for the arachidonic acid metabolism assays (below). The remaining S9 fractions were centrifuged at 100,000 × g for 60 min at 4°C. The resulting pellet yielded microsomes and the supernatant yielded cytosol (Lake, 1987). Additional epididymides were dissected from untreated animals, and sperm was collected from the cauda of the epididymis, as described previously (Filler, 1993). Sperm were washed in 0.1 M sodium phosphate buffer (pH 7.4) containing 50 μg/ml gentamycin sulfate and gently pelleted (500 × g, 5 min). Sperm were resuspended in buffer and homogenized

by hand with a glass and Teflon mortar and pestle. Total protein was quantified on all samples using the bicinchoninic acid method (Sigma Chemical Co.) with a 10 mg/ml BSA standard, according to the manufacturer's directions. Aliquots of the samples were frozen at -80^0C and stored for enzyme assays (below).

Gel electrophoresis and Western blotting. Proteins were separated on a 12% sodium dodecyl sulfate (SDS) polyacrylamide gel according to Laemmli (1970) and transferred to 0.45 μm nitrocellulose membranes using a wet transfer apparatus (Bio-Rad, Hercules, CA). Membranes were blocked with 5% nonfat dry milk (w/v) in Tris-buffered saline with 0.05% Tween-20 (v/v). The presence of mEH was detected by immunoblotting membranes with a 1:500 dilution of goat anti-mEH antibody. For detecting sEH, a 1:250 dilution of rabbit anti-sEH antibody was used. All membranes were subsequently incubated with alkaline phosphatase–conjugated donkey antigoat or goat antirabbit IgG (1:20,000 dilution). Immunoreactive proteins were visualized using Western Blue alkaline phosphatase substrate (Promega). A recombinant sEH standard was separated electrophoretically, blotted, and detected with a 1:3000 dilution of sEH antibody and a 1:20,000 dilution of alkaline phosphatase–conjugated goat antirabbit IgG. The sEH standard served as a molecular weight marker and positive control for sEH and to determine if there was any cross-reactivity between the anti-mEH antibody and the soluble form of EH. Duplicate blots incubated with appropriate concentrations of normal goat serum or normal rabbit serum in place of the primary antibody served as negative controls.

Determination of epoxide hydrolase activities. All substrates were synthesized in the laboratory as previously described (Borhan *et al*., 1995; Gill *et al*., 1983); sEH activity was determined using racemic [^{3}H]-*trans*-1,3-diphenylpropene oxide (tDPPO) as the substrate (Borhan *et al*., 1995). Briefly, 1 μl of a 5 mM solution of tDPPO in DMF was added to 100 μl cytosol (tissues) or homogenate (sperm) in sodium phosphate buffer (0.1 M, pH 7.4) containing 0.1 mg/ml BSA ([S] final = 50 μM). The mixtures were incubated for 30 min at 30^0C, and the reaction was quenched by the addition of 60 μl methanol and 200 μl isooctane. Incubations were vortexed vigorously to extract tDPPO into the isooctane, leaving the diol metabolites in the aqueous phase. Radioactive diol metabolites were quantified by liquid scintillation counting; assays were performed in triplicate. Endogenous glutathione and GST can interfere with the determination of sEH since glutathione conjugates may be formed from the tDPPO epoxide substrate (Gill *et al*., 1983). To account for radioactivity in the aqueous phase derived from the GST pathway, parallel incubations were made using 1 μM cyclohexyl-dodecyl urea in DMF to inhibit sEH activity (Morisseau *et al*., 2002). The resulting level of GST activity was subtracted from sEH activity levels. Microsomal EH activity was measured in essentially the same manner with [^{3}H]-*cis*-stilbene oxide (cSO) as the substrate in 0.1 M sodium glycine buffer (pH 9.0; Gill *et al*., 1983). Zero-protein and zero-time incubations served as blanks. Resulting counts per minute (CPM) were converted to pmol product and averaged over triplicate incubations. Specific activities were expressed in pmol/min/mg protein.

In vivo induction of sEH. Male rats (n = 4 per group) were administered either clofibrate (0.5% w/w) in diet or control diet for 10 days (Moody *et al*., 1986). Animals were sacrificed by CO_2 inhalation. Livers, testes, and epididymides were dissected, weighed, and processed for measurement of sEH enzyme activity, as described above.

Arachidonic acid metabolism. Arachidonic acid–derived epoxides and diols were measured in S9 fractions prepared from the liver, kidney, and epididymides from untreated rats. Briefly, the samples were suspended in 100 μl of 0.1 M sodium phosphate buffer (pH 7.4) and incubated (60 min, 30°C) with arachidonic acid (1 μM) and an NADPH-regenerating system in the presence or absence of the sEH inhibitor cyclohexyl-dodecyl urea (1 μM in DMF). Reactions were halted with the addition of 100 μl MeOH and centrifuged to remove the protein precipitate. Duplicate incubations were spiked with analytical surrogates, and arachidonate oxidation products in the isolated supernatant were separated by reverse-phase HPLC and detected using tandem mass spectroscopy, as described previously (Newman *et al*., 2002).

Statistical analyses. Group means were evaluated by two-tailed Student's t-tests and considered significantly different if $p < 0.05$. Analysis of variance (ANOVA) was used to detect significant differences in enzyme activity levels of different tissues and background (STATA, Stata Corp., College Station, TX). Differences in means were considered significant at $p < 0.05$.

RESULTS

mEH in the Rat Epididymis and Sperm

The microsomal form of EH was detected in the liver (Fig. 2, lane 1) at an apparent molecular weight of ~50 kDa. This band corresponded in size to mEH previously detected in the liver (Wang *et al*., 1982). A band corresponding to mEH was also detected in kidney microsomes, although it was visually less distinct than the protein band in the liver (Fig. 2, lane 2). Protein bands consistent in size with mEH were detected in the caput, corpus, and cauda of the epididymis (Fig. 2, lanes 3–5). However, no immunoreactive bands were detected in sperm (Fig. 2, lane 6). The polyclonal mEH antibody did not cross-react with the sEH recombinant protein, and no proteins consistent with mEH were detected on membranes incubated with normal serum in place of the primary antibody (data not shown). Several higher molecular weight bands (~60–80 kDa) were detected in each segment of the epididymis as well as in the liver and kidney. These most likely represent nonspecific cross-reactivity of the polyclonal antibody, as stated in the manufacturer's specifications. Additionally, purified rat liver mEH can aggregate to form a 70–80 kDa species (Guengerich and Davidson, 1982). It is possible that some of the immunoreactive bands represented a similar mEH aggregate in these samples.

To further confirm the presence of mEH in the epididymis, a substrate-based enzyme assay using cSO as the epoxide substrate was used to determine the catalytic activity of mEH in epididymal microsomes (Table 1). Because the Western blot results were similar across the caput, corpus, and cauda of the

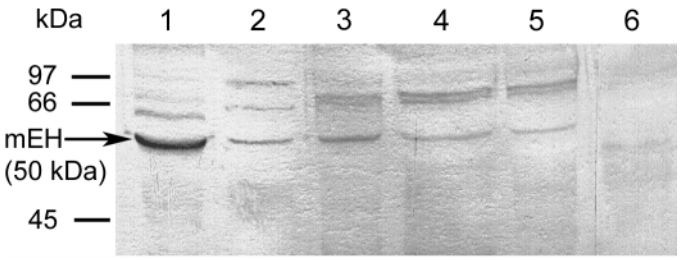

FIG. 2. Western blot analysis of microsomal epoxide hydrolase (mEH) in tissues from untreated male Sprague Dawley rats. A protein band (~50 kDa) was present in liver microsomes (lane 1) that corresponded to the previously published molecular weight for mEH. An immunoreactive band was detected in the caput, corpus, and cauda of the epididymis (lanes 3–5) as well as in kidney microsomes (lane 2), which was of similar molecular weight. No band corresponding to mEH was detected in whole sperm homogenate (lane 6). Nonspecific cross-reactivity was detected in lanes 1–5 at molecular weights of 60–80 kDa, as indicated in the manufacturer's specifications. Total protein loaded was 25 μg; numbers represent relative molecular masses of protein standards; blot is representative of four replicates.

TABLE 1
Epoxide Hydrolase Activities Measured in Tissues from Untreated Male Sprague Dawley Rats

	Specific activity (pmol product/min/mg protein)	
Tissue type	mEH activity microsomes	sEH activity cytosol
Liver	5700 ± 600*	1000 ± 317*
Kidney	—	2731 ± 485*
Epididymis	190 ± 20†	89 ± 12[a]†
Testis	510 ± 50*	—
Sperm	—	[b]

Note. Microsomal epoxide hydrolase (mEH) activity measured by hydrolysis of [^{3}H]-*cis*-stilbene oxide as the substrate, as described in the text. Values are reported as mean ± SD; $n = 3$ per group. Soluble epoxide hydrolase (sEH) activity measured by hydrolysis of racemic [^{3}H]-*trans*-1,3-diphenylpropene oxide as the substrate, as described in the text. Values are reported as mean ± SD; $n = 3$ per group. Activity less than 10 pmol/min/mg protein was considered not significant from background for the mEH and sEH assays.

[a]Originally sEH activity in the caput, corpus, and cauda of the epididymis were determined separately in $n = 3$ animals per group; the measurements were not statistically different across the segments; therefore, the nine observations were combined and reported as mean ± SD.

[b]Product level (18 ± 6 pmol/min/mg protein) approached the limit of detection for the assay (≅ 10 pmol/min/mg) and could not be distinguished from background.

*Indicates significantly different from background ($p < 0.001$, one-way ANOVA).

†Indicates significantly different from background ($p < 0.01$, one-way ANOVA).

epididymis, mEH activities were tested in microsomes prepared from the whole epididymis. The hydrolysis of cSO to the corresponding diol is specific to mEH (Ota and Hammock, 1980). In the present study, it was found that the microsomal rate of hydrolysis of cSO to the diol product was different for tissues, with liver > testis > epididymis. While the rate of product formation in epididymal microsomes (190 ± 20 pmol/min/mg protein) was only 3% of that measured in the liver, it was well above the assay limit of detection (10 pmol/min/mg protein).

sEH in the Rat Epididymis

Results from Western blotting suggested that the rat epididymis contained sEH (Fig. 3), albeit at low levels compared to the liver or kidney. Two adjacent protein bands (~65 and 62.5 kDa) were detected in cytosolic fractions prepared from the caput, corpus, and cauda of the epididymis (Fig. 3, lanes 3–5). The ~65 kDa band corresponded in size to the recombinant sEH standard (Fig. 3, lane 1) and to immunoreactive bands in the liver, testis, and kidney cytosol (Fig. 3, lanes 2, 7, and 8). Immunoreactive proteins were also detected in sperm samples, although no band was consistent with sEH (Fig. 3, lane 6). It appeared that the sEH in the liver was a lower apparent molecular weight than the recombinant standard, but that might have been due in part to the very different amounts of protein loaded in adjacent lanes (50 μg liver cytosol vs. 0.05 μg sEH standard). No protein bands were detected on duplicate blots incubated with normal rabbit serum in place of the anti-sEH antibody (data not shown). In a previous study using Western blotting with the same antibody, sEH was detected in rat liver cytosol at an apparent molecular weight of ~62 kDa (Draper and Hammock, 1999). Secondary immunoreactive bands were also detected in the rat liver cytosol, which the authors suggested were potential breakdown products or the result of differential post-translational modification (Draper and Hammock, 1999). Likewise, in the present study, the epididymis contained several immunoreactive bands that differed in molecular weight from sEH, possibly indicating nonspecific binding of the sEH antibody. Because of the number of nonspecific bands detected with the primary antibody, these data are only suggestive of the presence of sEH protein.

sEH Activity in the Rat Epididymis

A substrate-based assay specific to sEH was used to confirm that the epididymis contained sEH. Detectable sEH activity was measured in all segments of the rat epididymis using tDPPO as a substrate. There was no significant difference in enzyme activity for the caput, corpus, and cauda of the epididymis, consistent with the similar band intensities in each segment detected by Western blotting. Therefore, all observations were combined and a single specific activity was reported for the epididymis (Table 1). The rates of sEH activity were kidney cortex > liver > epididymis > sperm, although sEH activity in sperm preparations was not quantifiably different

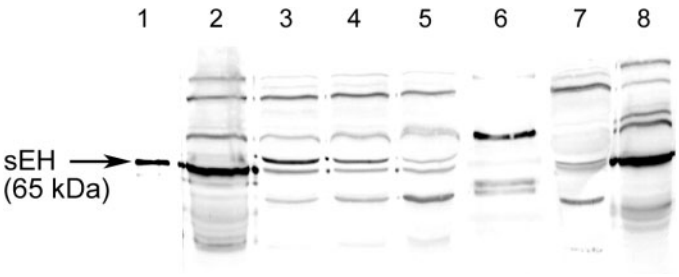

FIG. 3. Western blot analysis of soluble epoxide hydrolase (sEH) in tissues from untreated male Sprague Dawley rats. sEH was present as a dominant band at ~65 kDa in liver cytosol (lane 2) and kidney cortex cytosol (lane 8) and corresponded in size to the recombinant sEH standard (lane 1). Cytosolic preparations from the caput, corpus, and cauda of the epididymis (lanes 3–5) and the testis (lane 7) also contained immunoreactive bands that corresponded in size to the recombinant standard, although an adjacent immunoreactive band (~62.5 kDa) also appeared in each of those samples. A very faint 65 kDa band was visible in sperm homogenate (lane 6), although other protein bands were more immunoreactive. Total protein loaded was 0.05 μg in lane 1 and 50 μg in lanes 2–8. Primary antibody incubations in lanes 2–8 were 1:250 anti-sEH antibody. Primary antibody incubation for recombinant sEH standard was 1:3000 anti-sEH antibody. Blot is representative of six replicates.

from background. Activity less than 10 pmol/min/mg protein was considered not significant from background. The range of sEH activity measured in the Sprague Dawley rat liver was similar to levels previously measured in the male Wistar-Kyoto rat (690 ± 50 pmol/min/mg protein) using the same substrate (Yu *et al*., 2000). The liver sEH activities measured by Yu and colleagues (2000) and in the present study were at least an order of magnitude higher than the activity measured in the epididymis. Cyclohexyl-dodecyl urea, a well-characterized competitive inhibitor of sEH (Morisseau *et al*., 2002), was used to account for GST-derived conjugates of tDPPO. When this inhibitor was added to epididymal incubations, sEH activity was not detectable, supplying evidence of sEH-dependent metabolism of the substrate (data not shown).

Epididymal sEH Is Not Induced by Peroxisomal Proliferators

Peroxisomal proliferators are known inducers of sEH activity (Moody *et al*., 1986). In the present study, rats were administered a diet containing 0.5% (w/w) clofibrate, a well-characterized peroxisomal proliferator. Following a 10-day treatment, the levels of sEH activity in the liver, testis, and epididymis were compared for treated and control animals (n = 4 per group; Fig. 4). Specific activities for sEH in cytosolic fractions prepared from control rats were 57.2 ± 11, 43.4 ± 7.3, and 267.2 ± 46.2 pmol/min/mg protein for the testis, epididymis, and liver, respectively (mean ± SD). For the clofibrate-treated animals, there was no increase in sEH activity in either the testis (50.1 ± 3.4 pmol/min/mg protein) or the whole epididymis (48.2 ± 9.4 pmol/min/mg protein). However, there was a significant increase in sEH activity in the liver from treated animals compared to controls (968.2 ± 383 pmol/min/mg protein; Student's t-test, $p < 0.005$). These results are consistent with a previous study showing that clofibrate increased the level of sEH activity in the liver but not in the testis of treated rats (Moody *et al*., 1986).

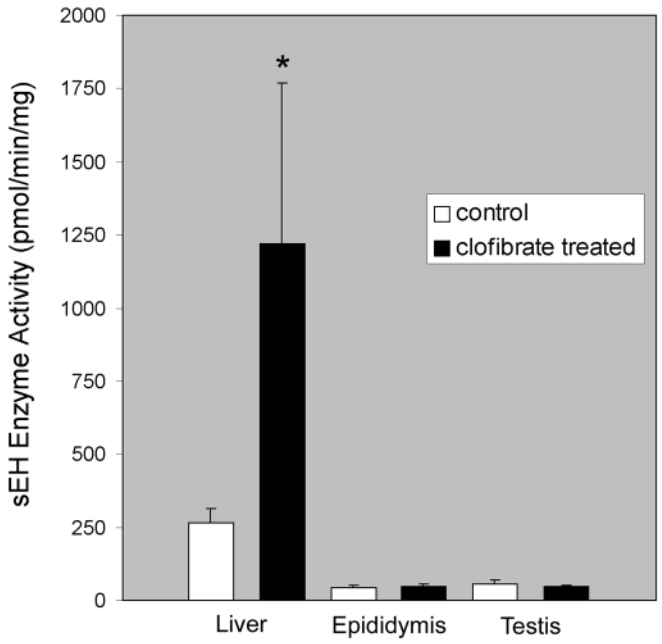

FIG. 4. Comparison of soluble epoxide hydrolase– (sEH-) specific activities in the testis, epididymis, and liver from clofibrate-treated and control rats (n = 4 per group). Data presented are mean ± standard deviation. Clofibrate treatment (0.5% w/w in diet for 10 days) significantly increased the rate of sEH enzyme activity in the liver (measured by the hydrolysis of [^{3}H]-trans-1,3-diphenylpropene oxide) compared to the liver control (two-tailed Student's t-test, $p < 0.05$). Clofibrate treatment did not result in significant changes in sEH enzyme activity in either the testis or epididymis.

The Epididymis Displays P450-Dependent Arachidonic Acid Metabolism

Arachidonic acids can be oxidized *in situ* by cytochrome P450 epoxygenases to arachidonic acid epoxides, i.e., EETs (Fang *et al*., 1997). EETs are substrates for sEH, which subsequently hydroxylates these epoxides to their corresponding DHETs (Chacos *et al*., 1983). In our study, a sensitive tandem mass spectroscopy method was used to detect EET and DHET metabolites of P450 epoxygenase and sEH metabolism (Newman *et al*., 2002). Results indicated that the rat epididymis was capable of forming both EETs and DHETs when incubated with exogenous arachidonic acid (Table 2). The most abundant metabolite formed by the epididymis was 14,15-DHET, detected at levels approximately one-third of the kidney. The levels of 8,9,- and 11,12-DHET were very low and could not be reasonably quantified above background. The products 5,6-EET and 5,6-DHET were not detected in epididymal incubations. The formation of DHETs can be inhibited by preincubating samples with the sEH inhibitor cyclohexyl-dodecyl urea prior to the addition of arachidonic acid. When cyclohexyl-dodecyl urea was added to epididymal S9 fractions, only the epoxides 11,12-EET and 14,15-EET were detected. The corresponding DHETs diols were not detected. Overall, the sum of EETs and DHETs formed by the epididymis was 5-fold and 15-fold lower than the kidney and liver, respectively. The lipoxygenase product 12-HETE was not detected in epididymal samples, however 5-HETE and 15-HETE were detected at levels comparable to those in the liver and kidney (Table 2). The cytochrome P450 product 20-HETE was not detected in any epididymal sample.

DISCUSSION

Epoxide hydrolases are an important class of metabolizing enzymes that are generally recognized as hydrolyzing toxic epoxides into their corresponding diols. The purpose of the present study was to investigate the presence and activity of the epoxide hydrolases in the epididymis. Both the microsomal and soluble forms of epoxide hydrolase were detected in the rat epididymis using immunochemical and biochemical tech-

TABLE 2
Comparison of the Specific Activity of Arachidonic Acid Oxidation in Tissues from Male Sprague Dawley Rats

Product	Liver (pmol product/min/mg protein)	Kidney (pmol product/min/mg protein)	Epididymis (pmol product/min/mg protein)	Percentage of epididymal activity[a]	
				Liver (%)	Kidney (%)
Cytochrome P450 epoxygenase product					
8,9-EET + DHET	267 ± 350	43 ± 35	< 50	—	—
11,12-EET + DHET	782 ± 670	372 ± 66	< 10[b]	—	—
14,15-EET + DHET	456 ± 360	153 ± 24	62 ± 41	14	40
Sum EETs + DHETs	1510 ± 1400	568 ± 70	109 ± 110	7	19
Lipoxygenase product					
15-HETE	601 ± 200	479 ± 190	345 ± 280	57	72
5-HETE	272 ± 50	301 ± 63	289 ± 170	106	96

Note. Arachidonic acid (1 μM) was incubated with tissue homogenates and products were quantified as described in the text. The tissue epoxygenase activity is the sum of the measured epoxide and diol regioisomers. Results for the kidney and liver are from three separate experiments. Activity in three segments of the epididymis (i.e., caput, corpus, and cauda) were individually assessed in triplicate but were not statistically different (Student's *t*-test, $p > 0.5$); therefore, all observations were combined. Reported results are the mean ± SD. DHET, dihydroxy eicosatrienoic acid; EET, epoxy eicosatrienoic acid; HETE, hydroxy eicosatetraenoic acid.

[a] Activity of epididymis arachidonic acid oxidation calculated as percentages of total liver or kidney arachidonic acid oxidation.

[b] 11,12-EET was observed near the detection limit in two of the nine observations.

niques. Western blotting detected mEH throughout the epididymis and results from the cSO-based enzyme assay suggested that epididymal mEH was metabolically active. However, compared to the testis and liver, epididymal mEH–specific activities were much lower. Observations from Western blotting also suggested that the epididymis contains sEH. Results from the hydrolysis of tDPPO indicated that epididymal sEH was metabolically active and could be inhibited *in vitro* by cyclohexyl-dodecyl urea (Morisseau *et al*., 2002). However, the overall rate of epididymal sEH activity was lower than activities measured in the liver and renal cortex. While our results suggest that epididymal EH affords some level of protection against toxic epoxides, the comparatively low levels may make the epididymis more reliant on other biomolecules, such as glutathione and GST, to detoxify epoxides and protect against toxicity.

Peroxisomes are cellular organelles involved in the oxidative degradation of fatty acids (Grant *et al*., 1994). Clofibrate is considered a classic peroxisomal proliferator (Hammock and Ota, 1983) that can also induce the activity of sEH (Grant *et al*., 1994). Following *in vivo* dosing with clofibrate, hepatic sEH activities were significantly elevated, consistent with previous reports (Loury *et al*., 1985; Moody *et al*., 1986). Peroxisomes have been described in the epididymis (basal and principal cells) and the efferent ductules (Reisse *et al*., 2001), and they are thought to be involved in the detoxification of peroxides generated during epididymal endocytosis or in the processes of lipid biosynthesis (Arrighi *et al*., 1994; Reisse *et al*., 2001). Our research indicated that clofibrate could not induce sEH activity in the epididymis. Peroxisomes have also been localized to the Leydig cells of rodent testes (Reddy and Svoboda, 1972), which is also the location of epoxide hydrolases (Ishii-Ohba *et al*., 1984). As with the epididymis, previous studies have not detected an increase in sEH activity in the testis following *in vivo* clofibrate treatment (Loury *et al*., 1985; Moody *et al*., 1986).

In addition to clofibrate, the compounds ciprofibrate and WY-14,653 induce peroxisome proliferation. All three compounds are associated with increased sEH activity in the livers of treated mice (Lundgren *et al*., 1988). Compared to ciprofibrate or WY-14,653, clofibrate is a relatively weak peroxisome proliferator (Reddy *et al*., 1982). It is possible that, if the duration of clofibrate dosing was increased or if ciprofibrate or WY-14,653 were used in place of clofibrate, sEH activity may have been induced in the testis or epididymis in the present study. However, in other studies, WY-14,643 did not induce peroxisome proliferation in the testis (Biegel *et al*., 1992) or alter the level of β-cell oxidation activity or proliferation in testicular cells (Biegel *et al*., 2001).

While our study only assessed the effect of clofibrate, it is possible that other compounds may alter sEH activity in the male reproductive tract. Previous work by Suzuki and Lee (1981) showed that the nematocide dibromochloropropprane, when given to animals as a single dose (125 mg/kg, po), increased sEH activity in the testis 40% over controls. Also, hypophysectomized rats fed a diet of 0.05% (v/w) perfluorooctanoic acid for 10 days had a significant (70%) increase in testicular sEH activity compared to sham-operated controls (Mehrotra *et al*., 1999). The authors found no concomitant increase in sEH activity from hypophysectomy alone (Mehrotra *et al*., 1999).

Steroid hormones may also play a role in the regulation of

epoxide hydrolase activity. Hormonal regulation of the epoxide hydrolases in reproductive tissues was first recognized by Mukhtar *et al*. (1978). They found that epoxide hydrolase activity in the rat testis rose significantly at the time of puberty (25 days after birth), coincident with increased testosterone synthesis (Mukhtar *et al*., 1978). A recent study found that the levels of epoxide hydrolase mRNA in the developing mouse testis start to increase between 15 and 20 days of age, rising to the highest level of expression after puberty and, again, paralleling the rise in testosterone within the pubertal testis (O'Shaughnessy *et al*., 2002). These studies only suggest that epoxide hydrolase activity is under the influence of hormonal regulation. However, Lee and colleagues (1980) showed a direct effect of hormones on testicular epoxide hydrolase. Rats treated for 5, 10, or 15 days with luteinizing hormone (LH) had significant increases in testicular EH–specific activity ($p < 0.01$; Lee *et al*., 1980). If the epoxide hydrolases in the testis are under hormonal regulation, it is possible that the same is true in the epididymis, an organ whose functions are regulated largely by hormonal interaction (Dyson and Orgebin-Crist, 1973).

While the endogenous role of epoxide hydrolases has yet to be fully elucidated, sEH in particular may have a role beyond xenobiotic metabolism in the epididymis. Specifically, because sEH mediates the hydrolysis of endogenous arachidonic acids, sEH in the epididymis may have a physiological function associated with the generation of arachidonic acid metabolites *in situ*. The kidney can be used as an example of the potential biochemical mechanisms of sEH beyond those of xenobiotic metabolism. For example, EETs and DHETs that arise via renal cytochrome P450 epoxygenase and sEH activity, respectively, act as modulators of ion and water balance (Zou *et al*., 1996). Cytochrome P450–derived EETs have been shown to inhibit renal Na^+-K^+-ATPases (Ominato *et al*., 1996) and alter Na^+ transport in the proximal tubule (Romero *et al*., 1991).

The epididymis and kidney share a similar embryologic origin (Howards, 1983). Specifically, the kidney proximal tubules and the proximal portions of the epididymis both arise from the mesonephric tubules (Howards, 1983; Robaire and Hermo, 1988). The epididymis, like the kidney, is comprised of a specialized epithelium that restricts the passage of specific ions, solutes, and macromolecules across cell boundaries (Hinton *et al*., 1995). Active Na^+-H^+ exchange, Cl^--CO_3- exchange, and a Na^+-K^+-$2Cl^-$ symporter all facilitate ion secretion into the epididymal lumen (Wong *et al*., 1980). The epididymal epithelia also contain active Na^+-K^+-ATPases, which establish a sodium gradient that moves water out of the lumen and concentrates sperm as they move from the proximal to the distal epididymis (Wong *et al*., 1980). The functional similarities of the kidney and epididymis support the notion that ion transport may be regulated by similar biomolecules in both organs.

Our results indicate that the epididymis is capable of forming both EETs and DHETs. The most abundant form detected was the 14,15-regioisomer at a level of approximately 40% of the kidney. This isomer has been shown to inhibit kidney Na^+-K^+-ATPases (Ominato *et al*., 1996). The potential interaction of the 14,15-regioisomer with ion transport in the epididymis could provide important insights into the regulation of this aspect of epididymal physiology and function. The epididymis also produced appreciable quantities of 5-HETE and 15-HETE at levels near that produced by the liver and kidney. These data suggest that there may be active lipoxygenases in the epididymis. To our knowledge, the function of lipoxygenases and these products of arachidonic acid metabolism in the epididymis have yet to be investigated.

We have shown, for the first time, that the epididymis contains metabolically active epoxide hydrolases; however, their activities were substantially lower than those in the liver or kidney. Epoxide hydrolases may contribute to the transformation of epoxides within the epididymis. However, GST-mediated metabolism of epoxides would be expected to be the primary metabolic pathway for the detoxification of epoxides because of their high enzymatic activity and abundance in the epididymis (Veri *et al*., 1994). Conversely, the soluble form of epoxide hydrolase along with cytochrome P450 epoxygenases have been shown to mediate the formation of physiologically important modulators of ion balance. It is possible that sEH and the arachidonic acid metabolites detected in the epididymis may be involved in ion balance in the epididymis.

ACKNOWLEDGMENTS

Portions of this report were presented at the 42nd Annual Meeting of the Society of Toxicology, Salt Lake City, Utah, March 9–13, 2003. Funding for this research was provided by the National Institute for Environmental Health Sciences (NIEHS) Center for Environmental Health Sciences (P30 ES05705), NIEHS (R37 ES02710), NIEHS Superfund Basic Research Program (P42 ES04699), NIEHS Center for Children's Environmental Health and Disease Prevention (1 P01 ES11269), and NIH/NIDDK UC Davis CNRU (P30 DK35747) Pilot Project. S. B. D. was supported in part by the NIEHS Training Grant in Environmental Toxicology (ES07059).

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